THE RESPIRATION OF SPISULA EGGS [1]

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Warburg, in 1908, found an increase in oxygen consumption after fertilization in the sea urchin egg. This seemed to support Loeb's theory (1906) of increased oxidative activities in connection with fertilization and development. Other workers subsequently found that eggs from other invertebrate animal groups showed no change at fertilization or even a decrease. It was eventually postulated that the change in oxygen consumption might be correlated with the stage of nuclear maturation at which fertilization occurs.

Whitaker (1933) removed the emphasis from oxidation as a determining mechanism in fertilization and, instead, posed the problem as one of understanding the differences in the metabolism of unfertilized and fertilized eggs. He found that unfertilized eggs of different species consume oxygen at very different rates, but that fertilization brings these rates closer together. Fertilization, therefore, seems to regulate the metabolism of the unfertilized egg to a level characterized by the more uniform needs of the developing organism.

Recently, Borei (1949) found that the change in oxygen consumption of sea urchin eggs at fertilization depends on the time after shedding at which fertilization occurs. The rate of the unfertilized egg gradually decreases, but the rate of the just-fertilized egg attains the same value regardless of the time elapsed between shedding and fertilization.

Biochemical studies on the nature and activity of the respiratory enzymes were conducted by many investigators, and especially by Runnström and his group. Runnström (1928a, 1928b, 1930) hypothesizes that colloidal rearrangements in the protoplasm, which are brought about by fertilization, change the spatial arrangement of the enzymes causing a change in the respiratory rate. Many protoplasmic changes in the egg have been observed in connection with fertilization (Runnström, 1949). Heilbrunn (1915, 1917, 1952) also connected changes in viscosity and other properties of the protoplasm, brought about by the calcium ion, to the events at fertilization.

Preliminary experiments with the egg of the surf clam, *Spisula solidissima* (Dillwyn), indicated that a decrease in oxygen consumption occurs after fertilization. It was thought that an examination of eggs from another animal group

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might help to determine which of the established respiratory properties of the sea urchin egg can be considered to represent general properties of an egg cell at fertilization, and which are merely special adaptations of the sea urchin group. Further measurements were made during the early development of the embryo.

<center>MATERIALS AND METHODS</center>

The surf clam, *Spisula solidissima* (Dillwyn), was obtained at the Marine Biological Laboratory at Woods Hole. The spawning season starts in the early part of June and lasts until late September. Fertilization of the eggs takes place externally and before the meiotic divisions have started. The first four cleavages are well synchronized and the embryos are swimming blastulae after 5½ hours at 21° C.

Eggs and sperm were obtained and handled according to the methods described by Allen (1951). Testes can be stored at 5° C. up to three days without impairing the fertilizing ability of the sperms. Washed eggs kept at 18° C. can be fertilized up to ten hours after removal from the ovary.
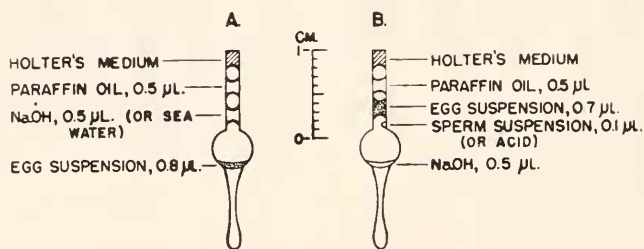

FIGURE 1. Cartesian diver charges. A. Loaded for O_2 uptake (or CO_2 output) measurements. B. Loaded for measurements during the course of which sperms or other substances are added.

The Cartesian diver respirometer was used in order to minimize crowding, to avoid damaging the eggs by shaking and to keep the cell number low in order to facilitate accurate cell counts. For measuring techniques see Linderström-Lang (1943), Holter (1943) and Borei (1948).

An egg suspension of 0.8 μl. was placed in the bulb of the diver. The first neck seal contained 0.5 μl. isotonic (0.47 M) NaOH, the second 0.5 μl. paraffin oil (Fig. 1). The control diver contained only sea water in addition to the seals. In order to facilitate loading and measurements and to avoid mechanical damage to the eggs by surface tension phenomena, the interior of the diver was coated with a hydrophobic layer of silicone. Either phenylsilicone (9989–1) or Dri-film (9987), manufactured by the General Electric Company, was used (Schwartz, 1949).

After measurements were completed, the eggs were removed from the respirometer and examined. Unfertilized eggs were tested for their fertilizability. The development of fertilized eggs was compared with that of control eggs kept in dishes in the respirometer water bath. Only those experiments were accepted in which unfertilized eggs showed over 95% fertilizability or fertilized eggs over 95% normal development.

The rate of development increases linearly between 18–23° C. At higher temperatures the rate of increase falls off. In order to secure a high respiratory rate, but at the same time not to operate too near the range of abnormal development, 21° C. was chosen for the experiments. The rate of development was determined by counting the number of nuclei and following their increase in number with time. Sections were made in order to study other embryological changes occurring.

For nuclear counts, the method described by Zeuthen (1951) for *Ciona* eggs was found much superior to whole mounts of fixed and Feulgen-stained eggs. Zeuthen's method consists of a careful and gradual compression of the eggs under a cover slip by withdrawing the suspension medium with tissue. The compression prevents further cleavage. Under the phase contrast microscope, the nuclei appear to be bright blue and are easily counted.

Sectioned material (10 μ) was obtained from eggs fixed in Zenker-formol, stained with toluidine blue and imbedded in paraffin.

RESULTS

Morphological changes during development

The morphology of the shed *Spisula* egg and its changes upon fertilization have been described by Allen (1953), who followed the events up to 100 minutes after fertilization. The present study corroborates his findings and extends the ob-
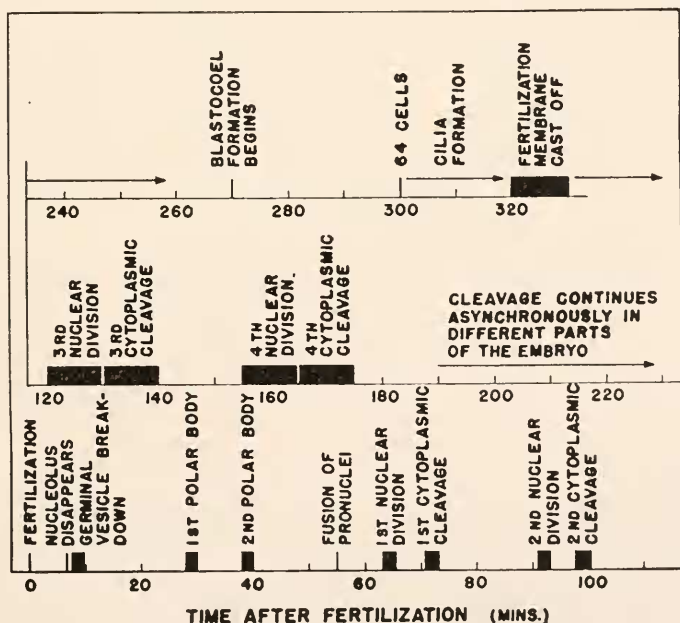

FIGURE 2. Development at 21° C. of *Spisula* eggs up to 330 minutes after fertilization. The width of the bands indicates the variation in time found for some of the respective events. The center of the band coincides with the time at which 50% of the embryos have completed the denoted event.

servations to 330 minutes after fertilization. Both studies were made at 21° C. The entire sequence is presented in Figures 2 and 3, in which the morphological events are related to time after fertilization. These eggs develop very synchronously up to the end of the second cleavage. It takes only two to three minutes for all of the embryos to complete a particular phase during this period. Thereafter, the synchrony between the individual cells in an embryo is less strict, but the embryos as a group continue to behave very synchronously.
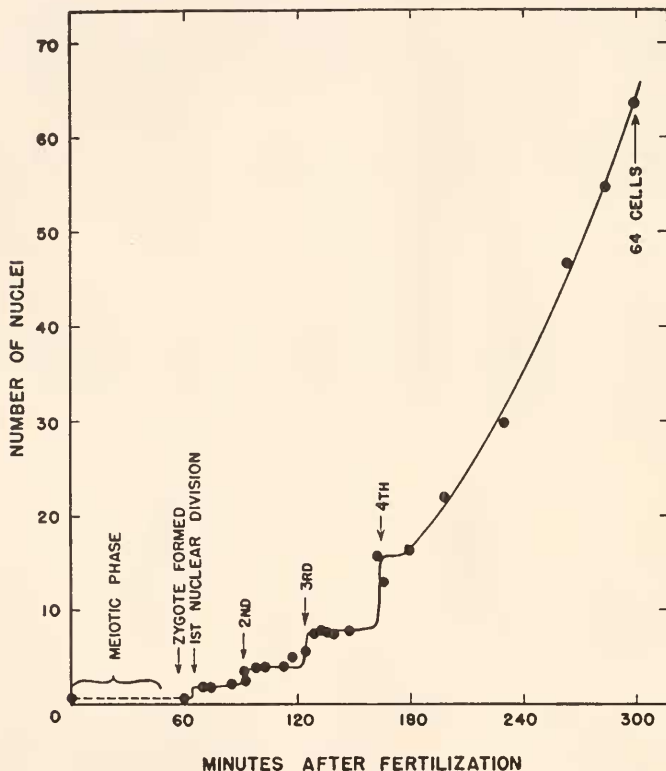

FIGURE 3. The increase in the number of nuclei during the early development of *Spisula* at 21° C.

Oxygen uptake

The oxygen consumption rate of 34 batches of unfertilized eggs was measured for periods up to eight hours after removal of the eggs from the ovary. Average values for $\frac{1}{2}$-hour intervals are given in Figure 4. If the curve, which is drawn according to the method of least squares, is interpolated back to zero time, the eggs have a mean rate of 3.8×10^{-5} μl. $O_2 \times egg^{-1} \times hour^{-1}$ immediately upon shedding. After eight hours, the rate has dropped to 3.0×10^{-5} μl. and the Student's t-test shows the drop to be highly significant ($P < .001$). The deviations in the earlier part of the curve are probably associated with differences in time

elapsed between the removal of the eggs from the ovary and the loading of the diver. This time varied from 0.5–2.5 hours.

The oxygen consumption during early development up to the swimming blastula stage was measured on 25 batches of eggs. Difficulties were encountered in measuring the rate just after fertilization. Unsuccessful attempts were made to fertilize the eggs inside the diver. According to Metz and Donovan (1949), the fertilizin secretion of these eggs is of a very high order. Fertilizin is known to inhibit fertilization and, since the eggs for technical reasons have to rest in the diver for times up to one hour prior to fertilization, its secretion may thus have prevented the fertilization process. The fertilization, therefore, had to be performed before the eggs were loaded into the diver.
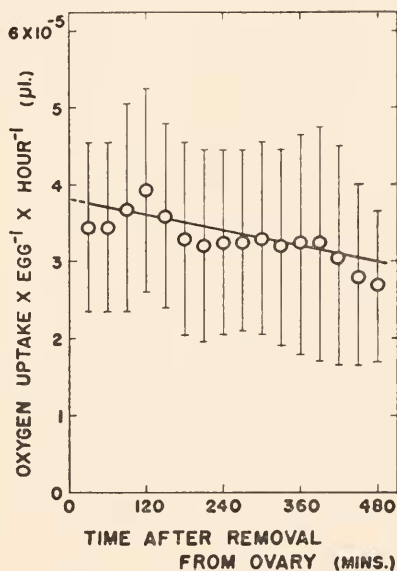

FIGURE 4. Rate of oxygen uptake at 21° C. of unfertilized *Spisula* eggs. Average values from 34 experiments. σ-values given for scattering.

Figure 5 shows the average values for ½-hour intervals after fertilization. Extrapolating back to ½-hour after fertilization gives a value of 3.0×10^{-5} μl. $O_2 \times$ egg$^{-1} \times$ hour^{-1}. A gradual increase is shown throughout the early cleavage stages up to the swimming blastula stage. The deviations in the early part of the curve may be due, as in the case of the unfertilized eggs, to the technical prehistory of the eggs and consequently to the different times at which measurements were begun. In most cases, loading was carried out immediately after fertilization and measurements begun after about ½-hour of equilibration. In some cases, however, loading was carried out after the first or second cleavage had taken place. The scattering recorded in Figure 5 is for the most part due to differences in rate between individual batches of eggs (biological scattering), rather than to spread between the points in each individual series of measurements.

The time at which eggs are fertilized after their removal from the ovary does

not affect the post-fertilization rate. The rates at 60 minutes after fertilization were plotted against the time after removal at which fertilization was performed, and a straight line was fitted to the values using the method of least squares. The t-test showed the slope of the line to be insignificant $(0.40 < P < 0.45)$.

Carbon dioxide output and respiratory quotient

The average of 11 experiments on the carbon dioxide output of unfertilized eggs gives the value 2.41×10^{-5} μl. $CO_2 \times egg^{-1} \times hour^{-1}$ for the time 200 minutes
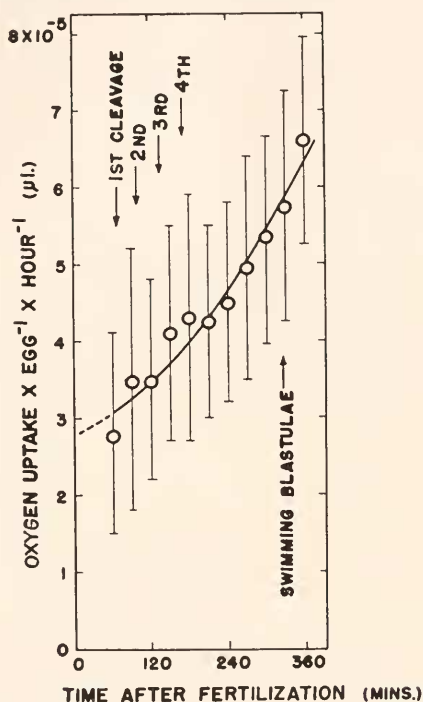

FIGURE 5.   Rate of oxygen uptake at 21° C. of fertilized *Spisula* eggs.   Average values from 25 experiments. σ-values given for scattering.

after removal from the ovary. The σ-value is 0.42. The R.Q. before fertilization is thus apparently much less than unity, probably in the vicinity of 0.7. It must be kept in mind that CO_2 measurements with the diver technique are inherently less accurate than O_2 uptake measurements. The reasons for this are two-fold: 1) leakage of CO_2 from the inside of the diver through the neck seals into the outside medium, and 2) errors introduced by the CO_2 retention properties of the eggs and sea water. Leakage through the seals was minimized in the experiments by use of an acid oil seal. Experiments on the retention properties indicate that the amount of CO_2 bound was not substantially changed during the time course of an experiment.

Nine runs on the CO_2 output after fertilization indicated that there is no perceptible change in the R.Q. value immediately after fertilization, but that later on a gradual increase toward unity seems to occur. The measurements comprise the time between fertilization and the swimming blastula stage.

DISCUSSION

A decrease in oxygen consumption rate of unfertilized eggs with time after removal from the ovary has been found by Holter and Zeuthen (1944) in *Ciona,* Zeuthen (1946) in *Rana* and Borei (1948) in *Psammechinus* and *Asterias.* In these cases, a constant rate was reached two to three hours after removal. In *Spisula,* the oxygen consumption rate of the unfertilized egg continues to decrease linearly for as long as eight hours after removal from the ovary. All of the above cited measurements were carried out with the Cartesian diver. No decrease in rate was found by Cleland (1950) in *Ostrea commercialis,* the rock oyster. Cleland employed the Warburg technique. It may be that the differences in technique could account for the differences in observations. However, unpublished data of Borei on sea urchins and of the author on *Spisula* indicate that Warburg and Cartesian diver measurements give similar results.

Cleland (1950) found no effect of fertilization on the oxygen consumption rate of *Ostrea commercialis.* Ballentine (1940), however, found a rise in respiratory rate at fertilization in the egg of *Ostrea virginica.* A large increase was also found by Ballentine (1940) in *Mactra lateralis.* In neither case did the latter author account for the time after removal of the eggs from the ovary at which fertilization took place. It is reasonable to assume, however, that one to four hours may have elapsed. The ratio in *Mactra* of the rates of fertilized eggs to unfertilized eggs is 1.8. Such a large value must indicate an increase in rate at fertilization over a long time span after shedding. *Mactra* and *Spisula* are very similar forms and have in common, among other things, the fact that the eggs are shed and fertilized in the germinal vesicle stage. In the oyster, however, the eggs proceed to the first maturation metaphase before they are fertilized. In the case of *Mactra* there is undoubtedly a rise in the respiratory rate at fertilization; in *Spisula,* there is a drop in rate at fertilization up to eight hours after shedding. The ratio obtained by Ballentine (1940) in *Ostrea virginicia* of 1.4 also indicates an increase in rate at fertilization.

Various workers (Shapiro, 1941; Tyler and Humason, 1937) have reported inconsistent results as to the direction and magnitude of the change in rate at fertilization in eggs of the same species and have ascribed this inconsistency to differences in individual batches of eggs. It is possible, however, that some of these differences could have been explained if the time of fertilization after removal of the eggs from the ovary had been taken into account. Recently Cleland (1950) tried to relate these differences to the kinetic state of the egg nucleus at the time of fertilization. This does not account, however, for the different behavior found in the two oyster species, *Ostrea virginica* (Ballentine, 1940) and *Ostrea commercialis* (Cleland, 1950) and in the two closely related molluscs, *Mactra lateralis* (Ballentine, 1940) and *Spisula solidissima.* The stage of meiosis at which the egg is normally fertilizable is probably not as important as the fact that both germinal vesicle breakdown and sperm entrance induce colloidal changes in the

egg. (*Cf.* review by Runnström, 1949.) It is well known that colloidal changes have a paramount importance for the activity of the cytochrome system (Keilin and Hartree, 1949; Borei, 1950, 1951).

The independence of post-fertilization respiration from the level before fertilization which was found by Borei (1949) to exist in *Psammechinus* was found to be the case in *Spisula* also. The mean rate at thirty minutes after fertilization is 3.0×10^{-5} μl. $O_2 \times egg^{-1} \times hour^{-1}$. It was previously shown that the mean rate of the unfertilized egg varies from 3.8×10^{-5} μl. $O_2 \times egg^{-1} \times hour^{-1}$ upon shedding to 3.0×10^{-5} μl. O_2 eight hours later. If the eggs are fertilized immediately after shedding or soon thereafter, there is a drop in the oxygen consumption rate within thirty minutes after fertilization. The longer the time interval between shedding and fertilization, the less is the drop in rate. After eight hours, there is no change in rate at fertilization. It might thus be that if the eggs stood for even longer periods of time than eight hours before being fertilized, there might be an increase in rate at fertilization. This range of possibilities was encountered in other material by Tyler and Humason (1937) as mentioned previously.

The rate of oxygen consumption of *Spisula* eggs during development follows the general pattern of other forms which have been studied. A gradual increase is observed (Fig. 5).

The carbon dioxide output measurements indicate certain conclusions as to changes in R.Q. during the development of the egg. These conclusions are in accordance with previous findings on echinoderm eggs and others. (For a review see Brachet, 1950.) Recently, Borei (unpublished) found in *Lytechinus pictus* that the R.Q. immediately after fertilization has a value of 0.7 or even lower, but that during the course of early development a gradual increase toward unity takes place. The findings in *Spisula* point in the same direction. It might thus be permissible to conclude that the egg immediately after fertilization draws on fat reserves, but gradually switches to carbohydrates later on. Analyses of the fat components of the embryo during different stages (Hayes, 1938; Hutchens, 1942; Öhman, 1944) also bear this out.

A comparison of the sea urchin and the clam egg respirations might permit the following general conclusions to be drawn:

1. The pre-fertilization respiration of a marine egg is a quantity determined by internal substrate conditions, and subject to a variation in magnitude dependent on the time elapsed from shedding.

2. On fertilization, however, more defined species-specific rates are predominant, *i.e.*, rates connected with the morphogenetic work that has to be done by the developing embryo.

3. The pre- and post-fertilization respirations seem to be independent processes. The prevailing rate of respiration before fertilization has no influence on the rate attained after fertilization.

4. The R.Q. results seem to validate the conclusion that different sources of substrate are utilized before and after fertilization. These conclusions may be considered to agree with a general concept that the egg before fertilization has to maintain only a physiological state making it ready and suitable for fertilization, whereas after fertilization new mechanisms come into play which have the sole purpose to provide for the morphological and functional development and differentiation.

I wish to express my appreciation to Dr. Hans Borei for his help in planning this problem and for his helpful advice and criticism during the course of the investigation.

SUMMARY

1. The early development of *Spisula solidissima* (Dillwyn) up to the swimming blastula stage is described.

2. The oxygen consumption of the unfertilized egg decreases gradually after removal from the ovary. The mean rate immediately after shedding is 3.8×10^{-5} μl. $O_2 \times egg^{-1} \times hour^{-1}$; eight hours later the value is 3.0×10^{-5}.

3. There is a decrease in oxygen consumption rate within 30 minutes after fertilization. The magnitude of this decrease depends on the time which has elapsed between the eggs' removal from the ovary and fertilization; the shorter the time, the larger the drop.

4. The post-fertilization oxygen consumption rate is independent of the rate before fertilization. Thirty minutes after fertilization, it has a value of 3.0×10^{-5} μl. $O_2 \times egg^{-1} \times hour^{-1}$.

5. The oxygen consumption rate of the fertilized egg increases gradually during development.

6. Carbon dioxide measurements indicate a respiratory quotient of 0.7 before fertilization. After fertilization, there is a gradual increase toward unity.

LITERATURE CITED

ALLEN, R. D., 1951. The use of *Spisula solidissima* eggs in cell research. *J. Cell. Comp. Physiol.*, **37**: 504–505.

ALLEN, R. D., 1953. Fertilization and artificial activation in the egg of the surf-clam, *Spisula solidissima*. *Biol. Bull.*, **105**: 213–239.

BALLENTINE, R., 1940. Analysis of the changes in respiratory activity accompanying the fertilization of marine eggs. *J. Cell. Comp. Physiol.*, **15**: 217–292.

BOREI, H., 1948. Respiration of oocytes, unfertilized eggs and fertilized eggs from *Psammechinus* and *Asterias*. *Biol. Bull.*, **95**: 124–150.

BOREI, H., 1949. Independence of post-fertilization respiration in the sea-urchin egg from the level of respiration before fertilization. *Biol. Bull.*, **96**: 117–122.

BOREI, H., 1950. Reactivation and protection of cytochrome oxidase preparations. *Biochem. J.*, **47**: 227–233.

BOREI, H., 1951. Post-fertilization respiration of the sea-urchin egg and the oxygen uptake of re-activated oxidase systems. *Science*, **114**: 684.

BRACHET, J., 1950. Chemical embryology. Interscience Publishers, Inc., New York, New York.

CLELAND, K. W., 1950. Respiration and cell division in developing oyster eggs. *Proc. Linnean Soc., N. S. Wales*, **75**: 282–295.

HAYES, F. R., 1938. The relation of fat changes to the general chemical embryology of the sea-urchin. *Biol. Bull.*, **74**: 267–277.

HEILBRUNN, L. V., 1915. Studies in artificial parthenogenesis. II. Physical changes in the egg of Arbacia. *Biol. Bull.*, **29**: 149–203.

HEILBRUNN, L. V., 1917. An experimental study of cell division. *Anat. Rec.*, **11**: 1–3.

HEILBRUNN, L. V., 1952. The physiology of cell division. From Modern trends in physiology and biochemistry. Academic Press, New York, New York.

HOLTER, H., 1943. Technique of the Cartesian diver. *C. R. Trav. Lab. Carlsberg, Sér. Chim.*, **24**: 399–478.

HOLTER, H., AND E. ZEUTHEN, 1944. The respiration of the egg and embryo of the ascidian, *Ciona intestinalis* L. *C. R. Trav. Lab. Carlsberg, Sér. Chim.*, **25**: 33–85.

HUTCHENS, W., 1942. Studies on cell metabolism and cell division. VI. Observations on glycogen content, carbohydrate consumption, lactic acid production and ammonia production of eggs of *Arbacia punctulata*. *J. Gen. Physiol.,* **24**: 717–731.

KEILIN, D., AND E. F. HARTREE, 1949. Activity of the succinic dehydrogenase-cytochrome system in different tissue preparations. *Biochem. J.,* **44**: 205–218.

LINDERSTRÖM-LANG, K., 1943. On the theory of the Cartesian diver microrespirometer. *C. R. Trav. Lab. Carlsberg, Sér. Chim.,* **24**: 333–398.

LOEB, J., 1906. Ueber die Hemmung der toxischen Wirkung hypertonischer Lösungen auf das Seeigelei durch Sauerstoffmangel und Cyankalium. *Pflüger's Arch. ges. Physiol.,* **113**: 487–511.

METZ, C. B., AND J. R. DONOVAN, 1949. Fertilizin from the eggs of the clam, *Mactra solidissima. Biol. Bull.,* **97**: 257.

ÖHMAN, L. O., 1944. On the lipids of the sea-urchin egg. *Arkiv Zool.,* **36A**: 1–95.

RUNNSTRÖM, J., 1928a. Zur experimentellen Analyse der Wirkung des Lithiums auf den Seeigelkeim. *Acta Zool. (Stockholm),* **9**: 365–424.

RUNNSTRÖM, J., 1928b. Struktur und Atmung bei der Entwicklungserregung des Seeigeleies. *Acta Zool. (Stockholm),* **9**: 445–499.

RUNNSTRÖM, J., 1930. Atmungsmechanismus und Entwicklungserregung bei dem Seeigelei. *Protoplasma,* **10**: 106–173.

RUNNSTRÖM, J., 1949. The mechanism of fertilization in metazoa. *Adv. in Enzymol.,* **9**: 241–327.

SCHWARTZ, S., 1949. The use of a silicone coating on the Cartesian diver microgasometer. *C. R. Trav. Lab. Carlsberg, Sér. Chim.,* **27**: 79–92.

SHAPIRO, H., 1941. Metabolism and fertilization in the starfish egg. *Biol. Bull.,* **81**: 278.

TYLER, A., AND W. D. HUMASON, 1937. On the energetics of differentiation, VI. Comparison of the temperature coefficients of the respiratory rates of unfertilized and of fertilized eggs. *Biol. Bull.,* **73**: 261–279.

WARBURG, O., 1908. Beobachtungen über die Oxydations-Prozesse im Seeigelei. *Zeitschr. Physiol. Chem.,* **57**: 1–16.

WHITAKER, D. M., 1933. On the rate of oxygen consumption by fertilized and unfertilized eggs. V. Comparisons and interpretation. *J. Cell. Comp. Physiol.,* **16**: 497–528.

ZEUTHEN, E., 1946. Oxygen uptake during mitosis; experiments on the eggs of the frog (*Rana platyrrhina*). *C. R. Trav. Lab. Carlsberg, Sér. Chim.,* **25**: 191–228.

ZEUTHEN, E., 1951. Segmentation, nuclear growth and cytoplasmic storage in eggs of echinoderms and amphibia. *Pubbl. Staz. Zool., Napoli,* **22** (Suppl.): 47–69.